

A Waterborne Behavioral Cue for the Actinotroch Larva of *Phoronis pallida* (Phoronida) Produced by *Upogebia pugettensis* (Decapoda: Thalassinidea)

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Abstract. *Phoronis pallida* (Phoronida) occurs as a commensal within the burrow of *Upogebia pugettensis* (Decapoda: Thalassinidea). *Upogebia*-conditioned seawater (UCSW) induced an exploratory swimming behavior in competent larvae of *P. pallida* in a dosage-dependent manner. This behavior included a significant increase in swimming speed that was directed downward, along with the repeated probing of the bottom with the sensory portion of the oral hood. The waterborne cue from the shrimp was present in the gut effluent, and the swimming behavior was not the result of the elevated ammonia concentration. Molecular weight separation of the UCSW estimated that the cue was between 10 and 50 kDa. Enzymatic treatments showed that the cue's activity could be eliminated by arginase and significantly reduced by lipase. Competent larvae were also induced to metamorphose when exposed to 20 mM CsCl for 30 min. Larvae did not respond to CsCl when cultured about 4 weeks past the onset of competence. Compared with actinotroch larvae of other phoronid species, *P. pallida* larvae exhibit greater behavioral specificity and neuronal differences within the hood sense organ. These anatomical and behavioral differences may have been maintained through a coevolutionary process among *P. pallida* and species of thalassinid shrimps that share *Upogebia* life-history characteristics.

Introduction

Many marine invertebrates produce larvae that spend hours to months in the plankton before becoming competent to respond to environmental signals emanating from the adult habitat (Pawlik, 1992; Rittschof *et al.*, 1998). These signals include a variety of physical and chemical cues. Investigations into chemical cues have focused mainly on those that induce metamorphosis. Such chemical cues may be waterborne or bound to the substratum. Some metamorphic cues are produced by conspecifics (Pearce and Scheibling, 1990; Zimmer-Faust and Tamburri, 1994; Matsumura *et al.*, 1998), prey species of the adult (Hadfield and Pennington, 1990; Lambert *et al.*, 1997; Krug and Manzi, 1999), or by bacterial and algal species associated with the substratum (Morse and Morse, 1991; Leitz and Wagner, 1993).

Larval recruitment has sometimes been described as a passive process dependent upon hydrodynamic forces (Harvey *et al.*, 1995), but increasing evidence suggests that the adult distribution of marine invertebrates can be influenced by larval behaviors (Gross *et al.*, 1992; Eckman *et al.*, 1994; Kingsford *et al.*, 2002). Some waterborne chemical cues emanating from the adult habitat induce changes in the swimming behavior and orientation of competent larvae before metamorphosis (Zimmer-Faust and Tamburri, 1994; Tamburri *et al.*, 1996). Although settlement behaviors have often been associated with the cessation of larval swimming (Rodriguez *et al.*, 1995; Zhao and Qian, 2002), other accounts describe settlement behavior that includes active habitat exploration before metamorphosis. Waterborne cues that induce exploratory behaviors are particularly influential in low-flow estuarine habitats during slack tide or periods of moderate flow (Krug and Zimmer, 2000; Browne and Zim-

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Abbreviations: FSW, filtered seawater; NCSW, *Neotrypaea*-conditioned seawater; UCSW, *Upogebia*-conditioned seawater.

mer, 2001; Fingerut *et al.*, 2003; Forward *et al.*, 2003). Some of these chemical cues are low-molecular-weight peptides with either arginine or lysine at their carboxy terminus. These small peptides are present in the effluent of invertebrates and produced as the result of invertebrate secretion, metabolism, and digestion (Rittschof, 1993; Browne *et al.*, 1998).

A diverse community of invertebrates inhabits the mud flats along the Pacific coast of the United States. A dominant feature of these habitats is the network of burrows created by the marine worms *Chaetopterus variopedatus* and *Urechis caupo*, and thalassinid shrimps such as *Neotrypaea californiensis* and *Upogebia pugettensis* (Ricketts *et al.*, 1985; Manning and Felder, 1991). These taxa are also known for the commensal symbionts that inhabit their burrows (Ricketts *et al.*, 1985). *U. pugettensis* has at least 15 documented commensal species associated with it, 7 of which are obligate relationships. Fewer commensals and obligate relationships are documented for each of the other three burrowing invertebrates (Haig and Abbott, 1980; Ricketts *et al.*, 1985; Hornig *et al.*, 1989). Studies of echinoderms and their symbiotic polychaetes have found that chemical cues from the host are recognized by the symbionts (Wagner *et al.*, 1979), and stimuli produced by the hosts probably attract particular commensal species at either the larval or the adult stage.

The Phoronida comprises at least 10 recognized species and a larval form known as the actinotroch (Emig, 1974, 1982). Species of phoronid are also noted for their wide geographical distributions and often occur in conspecific aggregations (Emig, 1982; Zimmer, 1991). Along the Pacific coast of the United States, adults of *Phoronis pallida* (Fig. 1A) are found embedded in the burrow wall of *Upogebia pugettensis* (Fig. 1B; also see Thompson, 1972). *U. pugettensis* incorporates mucus from its hindgut gland into the walls of its burrows (Fig. 1C). Thompson (1972) demonstrated that this mucus was composed of mucopolysaccharides that exhibit neutral, nonsulfated acid, weak-acid, and weak-acid-sulfated properties. This secretion binds the surrounding sediments and acts as a lubricant. Secretions from the hindgut gland and other properties of *U. pugettensis* may act as cues that enhance the recruitment success of competent *P. pallida* larvae.

This study investigated the behavior and metamorphosis of *Phoronis pallida* [(Schneider, 1862) Silén, 1952] larvae in response to possible cue sources from its adult habitat. Enzymatic treatments and molecular-weight separations were used to test whether behavioral cues for *P. pallida* larvae shared molecular properties with larval chemical cues from other marine invertebrates. These data are compared to treatments with compounds that artificially induce behavioral changes and metamorphosis in the actinotroch larvae of other phoronid species (Herrmann, 1979, 1995).

A waterborne cue produced in the effluent of *Upogebia*

pugettensis (Dana, 1852) induced competent larvae of *P. pallida* to swim faster in a downward direction and to repeatedly probe the bottom with the sensory portion of the oral hood. The induced behavior was dosage-dependent, and the activity of the cue was eliminated by treatment with arginase. The molecular weight of the cue was estimated to be between 10 and 50 kDa, indicating that the cue was not strictly a small peptide. Competent larvae were artificially induced to metamorphose when exposed to 20 mM CsCl for 30 min; however, no naturally occurring substrate or compound was found that induced natural metamorphosis. Although the exploratory behavior of *P. pallida* larvae differed slightly from reversible "settlement" behaviors described for veliger larvae (Chia and Koss, 1988), evidence suggests that active swimming behaviors ("dive-bombing") aid larvae in finding suitable metamorphic sites when in the bottom boundary layer (Finelli and Wethey, 2003). Compared with actinotroch larvae of other phoronid species, *P. pallida* larvae exhibit greater behavioral specificity and neuronal differences within the hood sense organ (Santagata, 2002). These anatomical and behavioral differences may have been maintained through a coevolutionary process among *P. pallida* and species of thalassinid shrimps that share life-history characteristics with *Upogebia*.

Materials and Methods

Collection of adults and culture of larvae

Phoronis pallida adults were collected in the summers of 1997, 1998, and 1999 from Bodega Bay (CA), Coos Bay (OR), and False Bay (WA). *P. pallida* was most often observed in the middle part of the Y-shaped burrow of *Upogebia pugettensis*. The best way to maximize the number of phoronids collected was to extract this portion of the shrimp's burrow by hand and sieve the sediment through a 1-mm screen. More than 50 phoronids per burrow have been observed in particular sites (Coos Bay, OR). Collection data supported the observations of Thompson (1972) that *P. pallida* is an obligate commensal with *U. pugettensis* but not with species of *Neotrypaea* at these study sites. Reproductive individuals of *P. pallida* (found May–October) are simultaneous hermaphrodites and may contain thousands of fertilized primary oocytes in the trunk coelom. Fertilized eggs are extruded through the nephridiopores into the burrow space, from which they are expelled to complete their development in the water column. Competent larvae were reared in the laboratory as described previously (Santagata, 2004).

Initial observations on the responses of larval stages to possible cues were made at the University of Southern California with larvae reared from adults collected from all field sites. Competent larvae are 450–550 μm in total length; they possess 10 tentacles and a single (red) corpuscle mass (Fig. 1D). Only this larval stage reared from

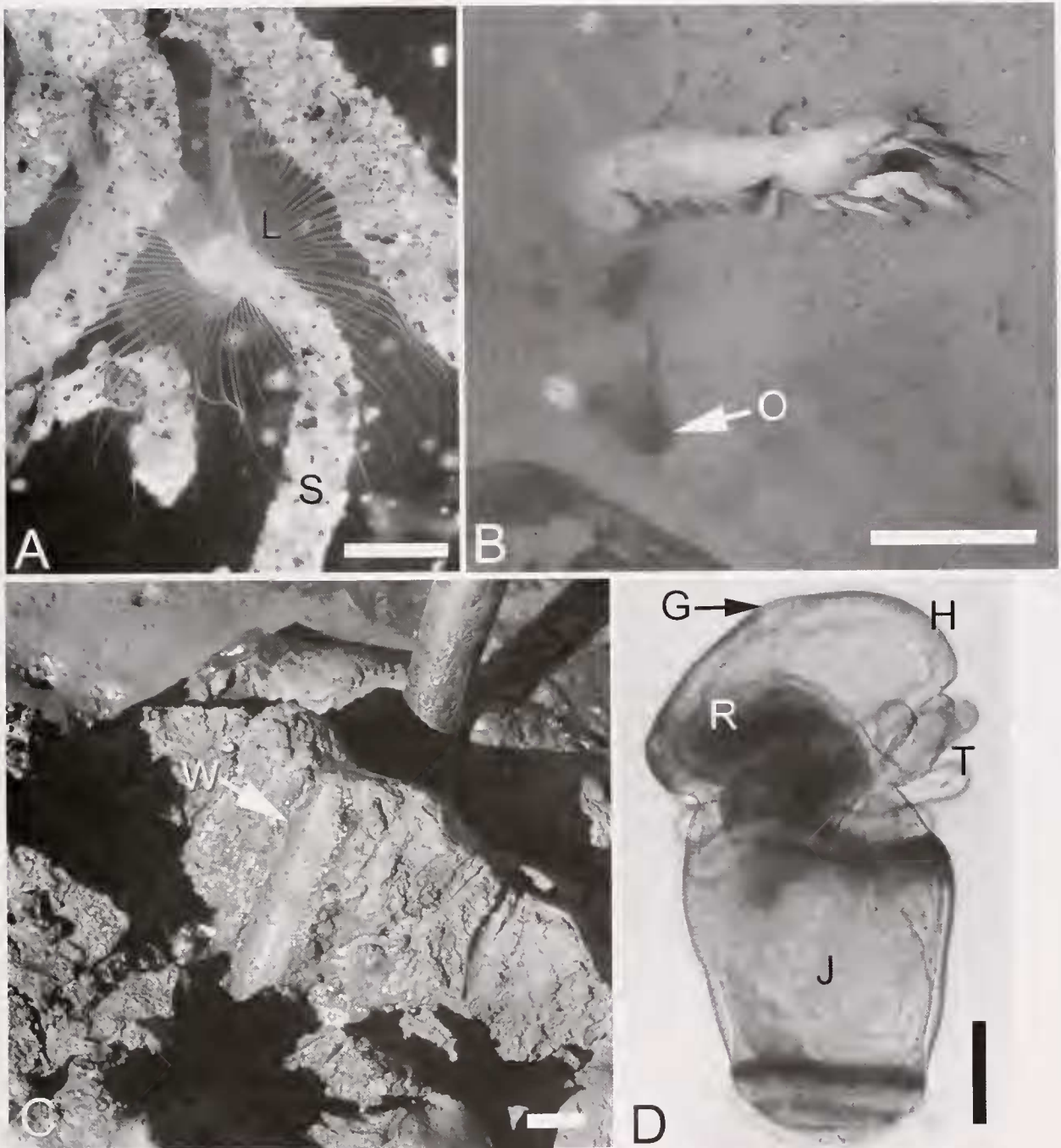


Figure 1. *Phoronis pallida* and *Upogebia pugettensis* from the populations at False Bay, San Juan Island, Washington. (A) Adult *P. pallida* with the lophophore (L) extended from its distinctive bent sand tube (S). (B) Adult shrimp of *U. pugettensis* extracted from its burrow. Note the opening to another *Upogebia* burrow (O). (C) Incurrent section of the burrow wall (W) of *Upogebia*. (D) Competent larva of *P. pallida* with a thickened hood (H) that includes the apical ganglion (G). Competent larvae also have a red corpuscle mass (R), 10 tentacles (T), and a differentiated juvenile trunk sac (J). Photograph in (D) reprinted with permission of Blackwell Publishing (Santagata, 2002). Scale bars: 2 mm (A), 5 cm (B and C), and 100 μ m (D).

adults collected from False Bay, Washington, was included for subsequent experiments carried out at Friday Harbor Laboratories, Washington.

Cue preparation and experimental assays

Upogebia-conditioned seawater (UCSW) was prepared by placing two adult shrimp (each about 10 cm in length) in 2 l of aerated, 0.22- μ m-filtered seawater (FSW) for 2 h at 15 °C. The shrimp were then removed, and the solution was refiltered (0.22 μ m). *Neotrypaea californiensis*-conditioned seawater was prepared in the same manner. The hindgut gland of one specimen of *U. pugettensis* was dissected out, and aqueous extracts were prepared in 5 ml of FSW using a mortar and pestle. The gut of another specimen was also dissected out and bisected sagittally. Aerobic bacteria were isolated from the gut tissue with a sterilized loop and cultured on a marine agar medium (80% FSW, 20% distilled water, 5 g peptone, 1 g yeast extract, and 15 g agar/l). Three morphologically different colonies from these plates were cultured individually in liquid medium (same as above minus the agar) in sterile test tubes for a day at 37 °C. One culture was inoculated with all three bacterial types. These cultures were spun down and resuspended in the same volume of FSW. Phoronid larvae were exposed to 1:10 dilutions of bacteria in FSW and aqueous extracts of the hindgut gland. Pieces of the carapace, gut tissue, and burrow walls of *U. pugettensis* were also tested as other possible cue sources with behavioral and metamorphic activity.

Assays were carried out within sterile, 6-well cell culture plates (BD Biosciences) with replicates of 5 or 10 larvae per well. Competent larvae of *Phoronis pallida* were removed from their culture vessels and placed in FSW for 2 h before use. Initial observations of the behavior demonstrated by competent larvae exposed to UCSW were that larvae swam faster in a downward direction and also probed the bottom with the apical portion of the oral hood. Larvae often spun around several times in one spot on the bottom before swimming away to probe other sites. This characteristic exploratory behavior was also described for competent actinotrochs of *Phoronis muelleri* (Silén, 1954) before the onset of metamorphosis. These behavioral traits were used as the criterion for whether a larva exhibited the swimming behavior within a given treatment. For a cue to be effective in flow, it should induce behavioral changes rapidly (Zimmer-Faust and Tamburri, 1994); therefore the total number of larvae exhibiting this behavior was counted within a 3-min interval. The minimum dosage of UCSW required to induce the majority of larvae to exhibit the swimming behavior was determined with a dilution range from 10 to 500. The relationship between dosage (\log_{10} -transformed) and percent of larvae behaviorally induced (arcsine-transformed) was determined with a linear regression. This was compared to the artificial induction of larval swimming

behaviors with elevated concentrations of ammonium chloride in FSW. Samples of UCSW solution were frozen and measured for total ammonia at the chemistry laboratory at the University of Washington's School of Oceanography.

Motion analysis of larval behavior

Horizontal swimming speeds over the bottom were estimated with point-to-point estimates from videotaped images of larvae in UCSW or FSW. Video images were gathered with a dissecting microscope and a Hitachi KP-C500 color CCD camera. Individual frames were captured from this videotape with a LG-3 frame grabber card (Scion Corporation) and processed with NIH Image software. Downward swimming velocities of larvae were measured with a 2-D motion analysis system (Motion Analysis Corp. model VP 110 and Expert Vision software, ver. 3.2) interfaced with a Sun Microsystems SPARC IPC computer workstation. Larvae were transferred to the top of a chamber (10 cm in width, 20 cm in length, and 31 cm in height) containing either FSW or a 1:10 dilution of UCSW. Trials were run at 16–18 °C. Larvae were not phototactic, and illumination was provided with a fiber optic light that pointed down the center of the chamber. Only larvae that remained within the cone-shaped illumination field could be visualized, eliminating larvae that traveled too close to the walls of the chamber. Larvae usually stayed within the light field for 30–90 s, and videotaped images of larval paths were sampled at 1–10 frames per s. The centroids of the raw paths were calculated; the resulting paths were edited for erroneous spurs, uniformly smoothed, and analyzed for their speed and trajectories.

Molecular weight of the behavioral cue

The molecular weight of the behavioral cue in UCSW was estimated with Millipore Centriplus concentrators with membrane cutoffs at 3, 10, 50, and 100 kDa. The concentrators were spun at 3000 g for the maximum time designated by the manufacturer. Once the concentrate was collected, it was diluted to its original concentration with FSW. Experimental trials were run as previously described with both the concentrate and filtrate at a 1:10 dilution. Positive controls consisted of recovery of the behavioral response by adding a 1:10 dilution of raw UCSW to all experimental trials after each treatment had been scored.

Enzymatic treatments of UCSW

To gain information about the chemical nature of the cue, UCSW was treated with various enzymes (see Table 2) according to the methods of Zimmer-Faust and Tamburri (1994), with some modifications. Concentrations of 2–4 units of enzyme per milliliter of UCSW were used, and incubations were at the optimum pH and temperature for

each enzyme for 30 min. Each solution of UCSW and enzyme was readjusted to 25 °C and a pH of 8.0 before being applied to the larvae (1:10 dilution). After swimming behavior in the enzymatic treatments was scored, untreated UCSW was added to each chamber at a dilution of 1:10 as a recovery-positive control to measure how many larvae within the enzymatic trials were capable of responding to raw UCSW. This also controlled for the unlikely possibility that the small amount of residual enzyme in these trials could have prevented the larvae from responding to UCSW. Incubations at these conditions of temperature and pH without enzyme have been shown to have no negative effects on the settlement cues of oysters (Zimmer-Faust and Tamburri, 1994). For these reasons, only the most extreme incubation conditions were tested for reducing the activity of UCSW without any enzyme (pH 5.0 or 9.5 at 37 °C for 30 min). One other remote possibility is that a particular enzyme might have the same effect on larval swimming behavior as UCSW. This would produce a false negative result even though the same enzyme might have degraded the activity of UCSW. Since there is no absolute way of knowing why a particular enzyme treatment did not work, this was not controlled for during these experiments. Enzyme treatments yield information only when they degrade the activity of the cue; when they do not have this effect, no information can be inferred about the chemical nature of the cue. Enzymatic treatments are only a rough guide about what the chemical nature of the behavioral cue might be rather than what it is not. Percentages of larvae exhibiting the swimming behavior in each treatment were arcsine-transformed before a one-way analysis of variance.

Artificial induction of metamorphosis

Metamorphosis was artificially induced by exposing larvae to 10–30 mM concentrations (in FSW) of either KCl or CsCl for no longer than 1 h. If a larva everted the trunk sac, it was immediately removed to FSW, and subsequent metamorphosis was observed through a dissecting microscope. Metamorphic stages were scored according to the following criteria, documented previously in Santagata (2002): stage one—partial histolysis of hood, telotrochal cells, and the larval portion of the tentacles; stage two—complete histolysis of larval tissues and partial eversion of the juvenile trunk sac; stage three—larval gut pulled inside the juvenile trunk sac, but portions of the larval trunk epithelium not completely pulled into the juvenile body; stage four—all previous events plus the larval trunk epithelium completely pulled into the juvenile body. All four stages could be completed within 2 h. A functional juvenile lophophore and circulatory system develops 2 days post-metamorphosis (Santagata, 2002). Most stage three and all stage four metamorphic types metamorphosed successfully, resulting in an anatomically complete juvenile.

Results

Behavioral cues

Live individuals of *Upogebia pugettensis*, dissected gut regions, burrow walls, and UCSW all induced the same behavioral response in larvae of *Phoronis pallida*, but did not induce metamorphosis (Table 1). Competent larvae exposed to both live specimens and freshly collected burrow walls also failed to induce metamorphosis (15 larvae exposed for 2 days). Aqueous extracts of the hindgut gland at a 1:10 dilution did not induce a behavioral response or metamorphosis. The three different bacterial colonies isolated from the gut of *U. pugettensis* did not induce any behavioral response; however most of these larvae did respond to UCSW (58% ± 17% SD). *Neotrypaea*-conditioned seawater (NCSW) produced inconsistent results. In six trials, no larvae responded to NCSW at a 1:10 dilution. Three of these larvae did respond behaviorally when exposed to full-strength NCSW, but did so after the 3-min period had elapsed. These same larvae did respond behaviorally to UCSW at a 1:10 dilution (35% ± 19 SD).

Since UCSW was the cue source with the most activity, all further experiments focused on characterizing it. Behavioral response to UCSW was correlated with the development of a 10th pair of larval tentacle buds and a red corpuscle mass. Development in culture is non-synchronous (Santagata, 2004), but the earliest development of these morphological traits and behavioral response to UCSW usually occurred between 30 and 35 days. Competent larvae responded to UCSW in a dosage-dependent manner (Fig. 2). Ten to thirty percent of larvae exhibited the swimming

Table 1

Effect of different possible cue sources for behavioral and metamorphic activity of competent larvae of *Phoronis pallida*

Cue source*	S/n
UCSW	5/5
Burrow walls	5/5
<i>Upogebia</i> gut tissue	5/5
<i>Upogebia</i> carapace	0/5
<i>Upogebia</i> gut bacteria	
Yellow bacteria	0/10
Orange bacteria	0/10
White bacteria	0/10
Bacterial mixture	0/10
<i>Upogebia</i> hindgut gland extract	0/30
NCSW 1:10†	0/60

None of these cue sources induced metamorphosis. Values are the number of larvae that exhibited the swimming behavior (S) out of the total number of larvae (n) in the treatment.

* UCSW and NCSW are, respectively, seawater conditioned with *Upogebia* and seawater conditioned with *Neotrypaea*.

† Three of these larvae did exhibit the swimming behavior when exposed to full-strength NCSW.

behavior in the presence of UCSW diluted as much as 1:500. However, most larvae exhibited the swimming behavior if exposed to a 1:10 dilution of UCSW. Most of the variation in the percentage of larvae that were behaviorally induced was explained by the dosage of UCSW ($r^2 = 0.84$).

I tested the responses of *Phoronis pallida* larvae to ammonium, which can induce settlement in oyster larvae (Coon *et al.*, 1990). The larvae responded behaviorally to very high levels of ammonium (10 mM; Fig. 3). However, UCSW contains only 5–7 μM of total ammonia, and levels during the experimental trials (1:10 dilution) were between 0.5 and 0.7 μM . According to the dosage-dependent response to ammonium chloride (Fig. 3), this concentration would be insufficient to elicit the same behavioral response.

Motion analysis

Average swimming velocities were 1.25 mm/s for larvae in FSW and 3.92 mm/s for larvae in UCSW. The maximum speed for most larvae exposed to UCSW was about 5.5 mm/s, but a few larvae reached speeds of 7 mm/s. A one-way analysis of variance between the two treatments shows a significant difference (Fig. 4, $df = 43$, F -ratio = 68.3, $P < 0.0001$). Once larvae reached the substratum, the increased swimming speed was maintained between probing sites (horizontal speeds, see Fig. 4, $df = 62$, F -ratio = 308, $P < 0.0001$). Overall, larvae in FSW swam more slowly and hovered at the top of the water column, and larvae in UCSW swam faster toward the bottom and probed the substrate (Fig. 5).

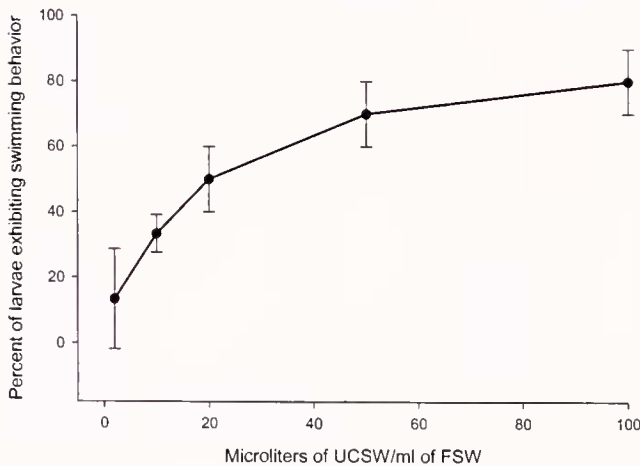


Figure 2. Dosage-dependent behavioral response of *Phoronis pallida* larvae to *Upogebia*-conditioned seawater (UCSW). Error bars equal one standard deviation from the mean and were calculated from 3 replicates of 10 larvae per treatment.

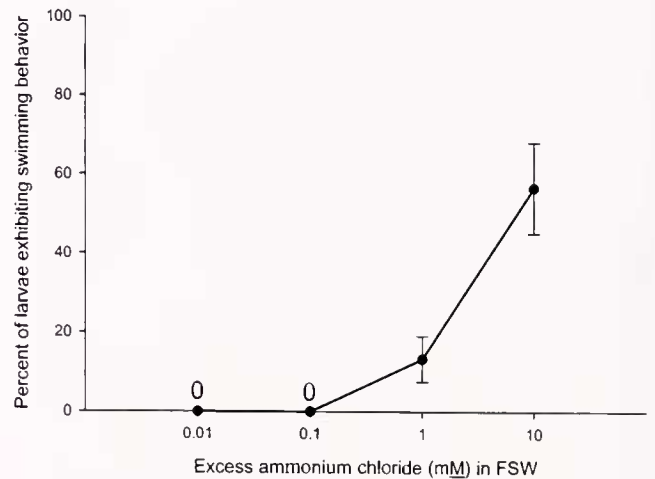


Figure 3. Behavioral response of *Phoronis pallida* larvae to increased concentration of ammonium chloride. Error bars equal one standard deviation from the mean and were calculated from 3 replicates of 10 larvae per treatment.

Molecular weight separation and enzymatic treatments of UCSW

The activity of UCSW fractions above 10 kDa (F -ratio = 1.1, $P > 0.34$) and below 50 kDa ($F = 0.73$, $P > 0.44$) was equal to that of untreated UCSW. The below 10-kDa and above 50-kDa fractions (F -ratio = 89.3, $P \leq 0.001$ and $F = 400$, $P < 0.001$, respectively) did not induce any changes in larval behavior, but these larvae did respond to raw UCSW (Fig. 6).

The most extreme enzymatic incubation conditions (5.0 and 9.5 at 37 °C for 30 m) had no effect on the activity of UCSW (7 of 10 and 8 of 10 larvae were induced, respec-

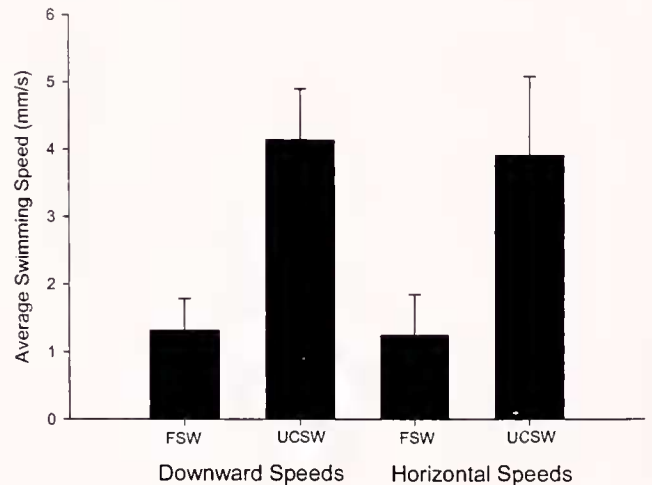


Figure 4. The average downward and horizontal swimming speeds of *Phoronis pallida* larvae in filtered seawater (FSW) and *Upogebia*-conditioned seawater. Error bars equal one standard deviation from the mean and were calculated from 30 paths per treatment.

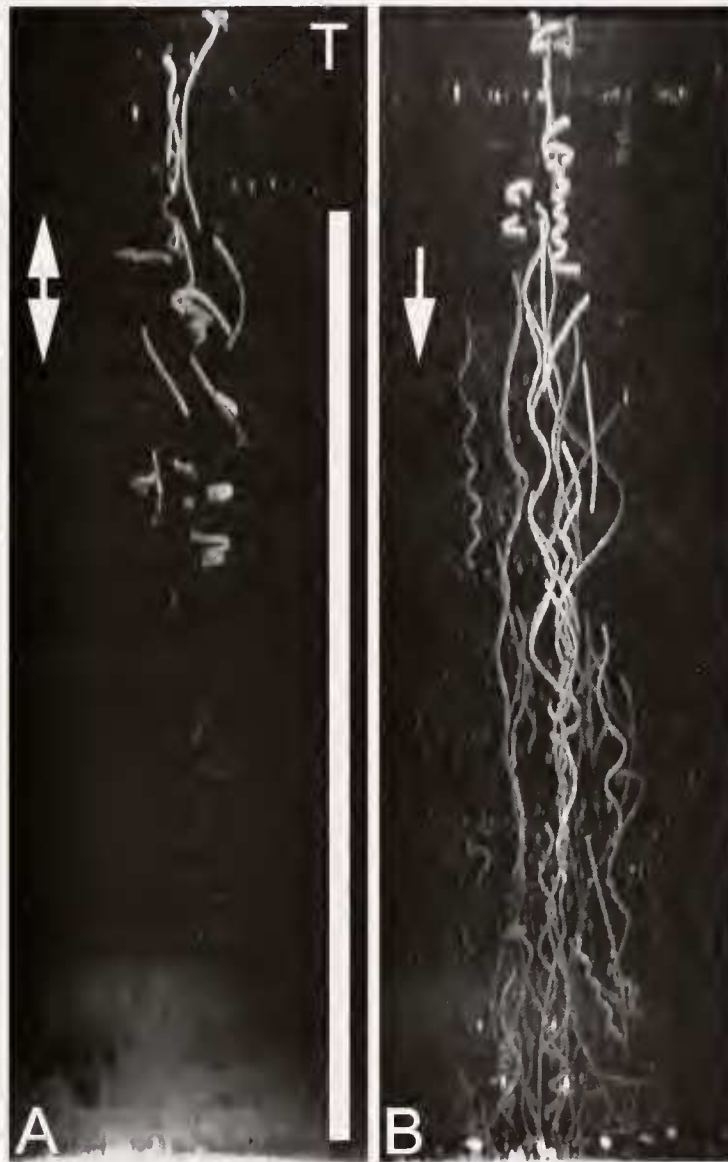


Figure 5. Time-lapse projections of larval behavior of *Phoronis pallida* in filtered seawater (FSW) and *Upogebia*-conditioned seawater (UCSW). Larval swimming was videotaped for 30 s. Video images were captured with iMovie 3.03, sampled at 10 frames/s, and exported as a stack of TIFF files. Time-lapse projections of these images were made with Image J 1.32 (Wayne Rasband, NIH). (A) Larval behavior in FSW. Larvae tended to hover at the top (T) of the chamber. (B) Larval behavior in UCSW. Larvae increased their swimming speed and swam down to the bottom of the chamber. Vertical scale bar is 20 cm.

tively). However, the arginase treatments consistently and completely eliminated the activity of the UCSW (Table 2, one-way ANOVA, $df = 28$, F -ratio = 63.9, $P < 0.001$). Lipase treatments also significantly reduced the activity of the UCSW, but about 13% of the larvae in this treatment exhibited the swimming behavior (one-way ANOVA, $df = 34$, $F = 27.9$, $P < 0.001$). Arginase specifically converts arginine to ornithine and requires that arginine be at the C-terminal position (Greenberg, 1960). Carboxypeptidase B should be able to cleave a C-terminal arginine, but this

treatment yielded only minor (albeit statistically significant, $F = 5.4$, $P < 0.05$) negative effects on the activity of UCSW. Minor negative effects were also observed with the sulfatase treatments ($F = 17.8$ and $P < 0.01$). Since about half of the larvae in each of the carboxypeptidase B and sulfatase treatments were still able to exhibit the swimming behavior, differences between these treatments and UCSW were judged nonsignificant. Carboxypeptidase P and prolidase treatments were tested in an attempt to overcome possible steric hindrances to carboxypeptidase B. Neither of

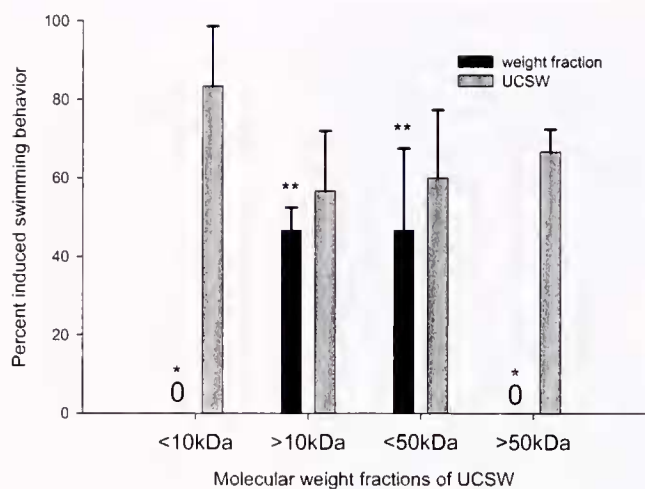


Figure 6. Results of the molecular weight fractions of *Upogebia*-conditioned seawater (UCSW) on the behavior of *Phoronis pallida* larvae. Error bars equal one standard deviation from the mean and were calculated from 3 replicates of 10 larvae per treatment. * Denotes where $P < 0.001$; ** denotes where $P > 0.34$.

these enzymatic treatments significantly reduced the activity of UCSW (Table 2).

Metamorphic induction with CsCl and KCl

The threshold concentration of cesium chloride that induced metamorphosis was approximately 10 mM, with an optimum effect at 20–25 mM (Table 3). CsCl at higher concentrations was toxic to larvae, and the minimum optimal exposure (20 mM) was chosen for all other

Table 2

Effect of enzymatic treatments of *Upogebia*-conditioned seawater on the swimming behavior of *Phoronis pallida* larvae

Enzyme	Replicates†	Percent \pm SD of larvae induced to swim‡	
		In enzyme-treated UCSW	After raw UCSW added
Arginase	15	0 \pm 0*	64.0 \pm 24.7
Carboxypeptidase B	9	43.3 \pm 18.0**	61.1 \pm 16.2
Carboxypeptidase P	6	66.7 \pm 12.1***	76.7 \pm 8.1
Carboxypeptidase Y	3	63.3 \pm 5.8***	73.3 \pm 5.8
Leucine aminopeptidase	6	46.7 \pm 21.6***	66.7 \pm 12.1
Prolidase	3	66.7 \pm 11.5***	76.7 \pm 11.6
Lipase	18	13.3 \pm 13.7*	56.1 \pm 23.8
Sulfatase	6	51.7 \pm 9.8**	75 \pm 8.4
Amylase and Maltase	3	63.3 \pm 20.8***	63.3 \pm 20.8
Lysozyme	6	70 \pm 8.9***	73.3 \pm 10.3

† Each replicate consisted of 10 larvae.

‡ UCSW, seawater conditioned with *Upogebia*. Statistically significant results are in bold type. Probability values are indicated as follows: * $P < 0.001$; ** $0.001 < P < 0.05$; *** $P > 0.05$.

Table 3

Effect of cesium chloride on metamorphosis of *Phoronis pallida* larvae

Concentration of CsCl in filtered seawater (in mM)	n	No metamorphosis	Stage			
			One	Two	Three	Four
5	15	15	0	0	0	0
10	29	23	2	1	2	1
15	27	15	3	3	6	0
20	30	2	12	6	10	0
25	30	3	10	9	7	1
30	31	12	12	5	2	0

The total number of larvae per treatment (N) is apportioned into metamorphic stages.

experiments. Behavioral response to CsCl was immediate, and larvae that everted the trunk sac did so 15–30 min after initial exposure. The number of metamorphic stages produced with CsCl through developmental time for a single larval culture is summarized in Table 4. At 5 weeks, CsCl induced predominantly early stages of metamorphosis. Most viable juveniles were produced between weeks 6 and 7. At week 9, these experiments resulted primarily in arrested metamorphic stages. By day 71, larvae were not able to complete any stage of metamorphosis when exposed to CsCl. Post-competent larvae also stopped feeding. In general, the development of metamorphic competence within culture is non-synchronous (data from 14 cultures, see Santagata, 2004), but viable juveniles resulted more often between weeks 5 and 9 under these culture conditions. Potassium chloride was toxic to larvae at concentrations from 10 to 30 mM, and these treatments did not induce muscle contractions or any stage of metamorphosis.

Table 4

Effect of 20 mM CsCl on ten-tentacle larvae of *Phoronis pallida* through developmental time

Age (days)	n	No metamorphosis	Stage		
			One	Two	Three and four
35 and 37	15	1	12	1	1
41 and 43	28	6	6	6	10
48, 49, and 50	33	3	8	6	16
63	20	5	14	0	1
68 and 69	14	2	9	1	2

Some close time points and metamorphic stages were pooled for convenience. n is the total number of larvae in each experiment.

Discussion

Behavioral responses to UCSW

In response to UCSW (seawater conditioned with the thalassinid shrimp *Upogebia pugettensis*), competent larvae of *Phoronis pallida* exhibited an exploratory behavior in a dosage-dependent manner. Larvae exhibiting this behavior swam fast and changed direction toward the bottom. Once in contact with the bottom, the larvae stopped for brief periods and probed the substratum with the apical ganglion (apical sense organ) and hood sense organ. Induced swimming behaviors of *P. pallida* larvae differ slightly from reversible "settlement" behaviors described for the veliger larvae of the nudibranch *Onchidoris bilamellata* (Chia and Koss, 1988). After reaching the bottom, *O. bilamellata* larvae crawl for up to 30 min unless they contact a barnacle (natural metamorphic cue). Some behavioral differences between veligers and actinotrochs are likely due to the functional morphology of their sensory and swimming structures. However, *P. pallida* larvae may also have maintained their increased swimming speeds between probing sites because of habitat differences. Barnacle habitats are large exposed surfaces, so when *O. bilamellata* larvae reach the bottom they are likely to be near a suitable place to metamorphose. In contrast, when the larvae of *P. pallida* reach the bottom, they must still get inside a burrow of *Upogebia*. Although pumping of the shrimp's pleopods may facilitate this event, *P. pallida* larvae may increase their chances of being swept into a burrow by maintaining their swimming speed between probing sites. Despite obvious differences in morphology, larval "settlement" behaviors similar to that of *P. pallida* have been described for oyster veligers (Finelli and Wethey, 2003). The dive bombing described for oyster veligers may aid the larvae in finding suitable metamorphic sites once in the bottom boundary layer (Finelli and Wethey, 2003), and it may represent a convergent behavioral response to waterborne cues among disparate larval forms.

Analysis of UCSW

Some naturally occurring settlement and metamorphic cues have been described as low-molecular-weight compounds (Hadfield and Pennington, 1990; Zimmer-Faust and Tamburri, 1994). Other studies have shown that larvae respond to insoluble, high-molecular-weight compounds that have smaller soluble components (Morse and Morse, 1991; Matsumura *et al.*, 1998; Krug and Manzi, 1999). In these latter studies, the cue was found to be a type of carbohydrate or protein. In this study, the stable waterborne cue was of high molecular weight with properties of both long-chain fatty acids and peptides.

Waterborne cues with lipid properties have been shown to act as phagostimulants and metamorphic inducers in

marine invertebrates. Unsaturated fatty acids from macroalgae that have been esterified into glycerolipids induce feeding in one species of abalone (Ando *et al.*, 1997). Similar chemical cues from the kelp *Egregia* also act as a feeding stimulant to a trochid snail (Wakefield and Murray, 1998). Glycoglycerolipids isolated from green algae induce metamorphosis in a sea urchin (Takahashi *et al.*, 2002). The role of free fatty acids as chemical cues to larval settlement remains ambiguous. In species where free fatty acids have been implicated, it is not clear whether these compounds were extraction contaminants or acted as secondary cell-signaling messengers (for review of these analyses and discussions, see Pawlik, 1990; Jensen *et al.*, 1990; Leitz, 1993; Kitamura *et al.*, 1994). Resolving the lipid-like properties of the behavioral cue in UCSW will require finer chemical analyses.

The complete degradation of the activity of UCSW by arginase points to an important peptide component. Removal of all low-molecular-weight compounds (less than 10 kDa) did not significantly reduce the activity of the UCSW. This suggests that the peptide component of the waterborne cue was bound to the lipid component. Taken together, the evidence is consistent with the cue having glycerolipid or peptidoglycan-like properties. However, the minor negative effect of the sulfatase treatments suggests other possibilities, such as a resemblance to the sugars produced by diatoms (Zimmer and Tamburri, 1994). Besides algae, another possible source of these compounds is the species-specific types of bacterial symbionts in the gut of thalassinids (Harris, 1993; Pinn *et al.*, 1999). At least two species of *Upogebia* enrich their burrow walls with organic matter, which serves as a good niche for bacterial colonization (Thompson, 1972; Kinoshita *et al.*, 2003). Bacterial cell walls are a source of peptidoglycan-like molecules, and some produced by gram-negative bacteria are particularly resistant to degradation in marine environments (Jorgensen *et al.*, 2003). These factors would make the settlement cues associated with the effluent and burrows of *Upogebia* distinct from those of other co-occurring thalassinids or any other species in the mudflat.

Natural and artificial induction of metamorphosis

Herrmann (1979, 1995) documented that the larvae of *Phoronis muelleri* and *P. psammophila* are naturally induced to metamorphose with gram-positive and gram-negative bacteria isolated from sediments found in the adult habitat. The threshold concentration of bacterial cues necessary to induce metamorphosis also decreased during the competency period (Herrmann, 1995). If the competency period is prolonged, competent larvae of *P. muelleri* and *P. psammophila* will eventually (spontaneously) metamorphose, but this often results in what Herrmann described as aberrant metamorphosis. Although not rigorously tested,

Herrmann's metamorphic models support the hypothesis that metamorphic specificity decreases with larval age. However, Toonen and Pawlik (2001a) found no support for the "desperate larva hypothesis" during the prolonged planktotrophic period of *Hydroides dianthus* larvae. I found the behavioral and metamorphic cues for *P. pallida* to be more specific than for other species of phoronids, and I never observed spontaneous metamorphosis in culture. Unfortunately, a naturally occurring metamorphic inducer has not been found for *P. pallida*, but the CsCl experiments suggest a competency period of about 4 weeks. Estimates of competency periods with natural and artificial inducers can yield different temporal patterns (Pechenik *et al.*, 1995). Furthermore, the onset and duration of metamorphic competence is also affected by food availability (Pechenik *et al.*, 1996). For these reasons, measurements of the competency period of *P. pallida* larvae are only a rough estimate under these culture conditions (see Santagata, 2004). Loss of the behavioral and morphogenetic abilities gained at metamorphic competence has been found in other planktotrophic larval types after similar competency periods (Avila, 1998; Toonen and Pawlik, 2001a). Behavioral specificity during metamorphic competence clearly has some life-history-specific, species-specific, and polymorphism-specific trends (Krug, 1998; Hadfield *et al.*, 2001; Toonen and Pawlik, 2001b). Overall, data contained here and in Santagata (2004) are more consistent with the findings of Toonen and Pawlik (2001a). This may represent a functional convergence in life-history traits among planktotrophic larvae that exhibit specific settlement preferences.

Consistent with the data from *Phoronis muelleri* and *P. psammophila* (Herrmann, 1979, 1995), excess Cs^+ but not K^+ induced metamorphosis in the competent larvae of *P. pallida*. Evidence suggests that excess Cs^+ and NH_4^+ induces larval metamorphosis by increasing levels of intracellular NH_4^+ , which in turn binds more methyl groups, which reduces the levels of S-adenosylmethionine (Berking, 1988; Berking and Herrmann, 1990). Interestingly, excess Cs^+ induced both the swimming behavior and metamorphosis, but excess NH_4^+ induced only the exploratory behavior. Metamorphic induction by excess Cs^+ and not K^+ or NH_4^+ may be indicative of signal transduction mechanisms that normally inhibit metamorphosis unless they are overwhelmed by external excitatory stimuli (Pires *et al.*, 2000; Leise *et al.*, 2001; Pechenik *et al.*, 2002; Katsukura *et al.*, 2003). This type of system would be advantageous for larvae that require specific behavioral and metamorphic cues.

Hood sense organ and behavioral specificity

Metamorphic competence in actinotrochs is defined by the differentiation of the juvenile neuromuscular system, development of the hood sense organ, and development of

neuronal connections between the larval and juvenile neuromuscular systems (Santagata, 2002). At least four phoronid species have serotonergic sensory neurons in their hood sense organs and are capable of spontaneous metamorphosis when collected from the plankton (Santagata and Zimmer, 2002). *Phoronis pallida* is different from these species in at least two ways: the sensory neurons in the hood sense organ are not serotonergic (Santagata, 2002); and spontaneous metamorphosis does not occur in culture during the competency period (Santagata, 2004). Developmental modifications of chemosensory circuits have been correlated with behavioral specificity in the nematode *Caenorhabditis elegans* (Melkman and Sengupta, 2004). Compared with the actinotroch larvae of other phoronid species, the larvae of *P. pallida* have greater behavioral and metamorphic specificity that corresponds with modifications of the neuronal cell types within the hood sense organ.

Thalassinid life-history characteristics

Pacific and Atlantic populations of *Phoronis pallida* exhibit differences in adult habitat. Atlantic populations occur in soft sediments along with species of thalassinid shrimps, but have not been found as commensals within thalassinid burrows (Viéitez and Emig, 1979; Silén, 1952). Hawaiian populations of *P. pallida* occur in sandy substrates with no mention of an association with thalassinids (Bailey-Brock and Emig, 2000). Although the distributions of *P. pallida* and *Callianassa limosa* are similar in Port Phillip Bay, Australia (Poore, 1975; Emig *et al.*, 1977), the only other species of thalassinid that definitively contains *P. pallida* as a commensal within its burrow is *Upogebia major* (see fig. 1C in Kinoshita, 2002), which occurs in Tokyo Bay, Japan. *U. major* produces a burrow similar to that of *U. pugettensis*, and the two species share several behavioral traits (Kinoshita *et al.*, 2003). Whether distant populations of *P. pallida* that occur in different adult habitats represents cryptic speciation remains to be tested.

Differences in abundance, feeding behavior, and physiology may also account for the diversity of commensal species found with species of *Upogebia* rather than with other thalassinid shrimps. *U. pugettensis* and *U. major* each occur in a mean density of 40 shrimp per square meter, with occasional abundances greater than 100 shrimp per square meter (Swinbanks and Luternauer, 1987; Dumbauld *et al.*, 2001; Kinoshita *et al.*, 2003). Abundances in these ranges would provide a suitable settlement target for recruiting commensal species. Species of *Upogebia* are primarily herbivorous suspension feeders that occasionally engage in deposit feeding, whereas most other genera of thalassinids depend more on deposit feeding or omnivorous scavenging (Griffis and Suchanek, 1991; Nickell and Atkinson, 1995; Coelho *et al.*, 2000). The greater reliance upon suspension feeding may increase the recruitment success of symbiont

larvae to *Upogebia* burrows. *U. pugettensis* is also less resistant to anoxia and reduced salinity than co-occurring species of *Neotrypaea* (Thompson and Pritchard, 1969; Torres *et al.*, 1977; Swinbanks and Murray, 1981; Swinbanks and Luternauer, 1987; Astall *et al.*, 1997), and thus it is limited to more environmentally stable habitats. Habitat stability may contribute to greater survivorship among commensal species that associate with thalassinid shrimps that have *Upogebia* life-history characteristics.

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